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Selection and Characterization of ColE1 Plasmid Mutants That Exhibit Altered Stability and Replication

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This report describes a method for isolating mutants of plasmid ColE1 that exhibit unstable maintenance and altered replication characteristics. It also describes the initial characterization of four mutants isolated by that method. A chimeric plasmid, pHSG124, containing a ColE1 derivative and a temperature-sensitive replication derivative of pSC101 was mutagenized in vitro, using hydroxylamine. By adjusting the growth conditions of transformants containing the mutagenized chimeric deoxyribonucleic acid, it was possible to rapidly screen colonies and identify those that had a high probability of carrying ColE1 mutants that exhibit unstable maintenance. Of those mutants, some exhibited altered copy number or accumulated catenated structures. Evidence is presented which suggests that the mutations in three of the mutants are probably located in the *Hae*II A fragment of ColE1.

The study of the mechanism of replication and maintenance of ColE1 would be facilitated if a simple selection of point mutants affecting those processes were available. A previously isolated chimeric plasmid, pHSG124 (9), was used to develop such a selection. pHSG124 was formed by ligating a ColE1 derivative, pDMS630 (9), which carries a Tn*3* transposon insertion, to a temperature-sensitive replication mutant of pSC101 (pHSG1) (9, 11). pHSG124 exhibits the replication properties of ColE1, which include a copy number of about 15 per chromosome, stable maintenance at 30 and 42°C, and continuing replication in the presence of chloramphenicol (9, 10). It also confers both ampicillin and tetracycline resistance phenotypes on its host. A deletion of the replication origin and parts of the replication region of the ColE1 component of pHSG124 changed the replication characteristics of the mutant chimera to those of pHSG1, which has a copy number of about four per chromosome, ceases to replicate at 42°C, and does not replicate in the presence of chloramphenicol (9, 10). Point mutations generated by hydroxylamine mutagenesis (7) that cause absolute or partial defects in the ColE1 replication element of the chimera should lead to the appearance of chimera instability at 42°C which can be monitored by observing the simultaneous segregation of the ampicillin and tetracycline resistance phenotypes conferred on the host cell by the plasmid (9, 10). It was reasoned that plasmid segregation could manifest itself by the production of small colonies on agar containing both ampicillin and tetracycline if cells transformed by mutant DNA were first grown on antibiotic-containing agar at a permissive temperature and then shifted to a nonpermissive temperature. Plasmids were isolated from such small colonies and shown to exhibit altered stability and replication characteristics. This paper describes the isolation and initial characterization of such mutants.

MATERIALS AND METHODS

Bacteria and plasmids. The strains of *Escherichia coli* used were P678-54 (*thr leu thi rpsT* Sm^r) (14), Om84 *tyr*(Am) *trp*(Am) *thy his ilv supD* (9), and HfrH5, obtained from B. Bachman. Each strain contained appropriate plasmids that were introduced by standard DNA transformation methods (see below). Plasmids used are shown in Table 1. pDMS630 is ColE1 containing a Tn*3* transposon that confers ampicillin resistance (9, 14). pDMS6642, is a deletion mutant that was derived from pDMS6-6d4-11 (18) (renamed pDMS6641) (9). It was formed by ligating a *Hae*II fragment obtained from pDMS6641 that contains approximately 850 base pairs of the ColE1 plasmid and the β-lactamase gene of the Tn*3* transposon with the *Hae*II E fragment of ColE1 (see Fig. 5). pHSG124 (9) is a chimeric plasmid formed by ligating *Eco*RI-cleaved pDMS630 to *Eco*RI-cleaved pHSG1, which is a temperature-sensitive replication mutant of pSC101 (9). Since pHSG1 exhibits temperature-sensitive replication, the pHSG124 chimera is only stable at 42°C if its pDMS630 component exhibits temperature-resistant replication (9). Mutant derivatives of pHSG124 that were isolated and are reported in this work are pDMS1020, pDMS1037, pDMS1043, and pDMS1050. The pDMS630 mutants isolated by cleaving those mutant pHSG124 chimeras with *Eco*RI and separately religating the pDMS630 components are

TABLE 1. *Plasmids used*

Plasmid	Drug resistance	Stability[c]		Colicin	
		30°C	42°C	Production	Immunity
ColE1		S	S	+	+
pDMS630	Ap[a]	S	S	+	+
pHSG124	Ap, Tc[b]	S	S	−	+
pDMS1020	Ap, Tc	S	U	−	+
pDMS1037	Ap, Tc	S	U	−	+
pDMS1043	Ap, Tc	S	U	−	+
pDMS1050	Ap, Tc	S	U	−	+
pDMS1020-6	Ap	U	U	+	+
pDMS1037-6	Ap	U	U	+	+
pDMS1043-6	Ap	U	U	+	+
pDMS1050-6	Ap	U	U	+	+
pDMS6641	Ap	S	S	−	−
pDMS6642	Ap	S	S	−	−

[a] Ampicillin resistant.
[b] Tetracycline resistant.
[c] S, Stable; U, unstable.

pDMS1020-6, pDMS1037-6, pDMS1043-6, and pDMS1050-6. pDMS1020-7 and pDMS1043-7 are plasmids formed by ligating the ColE1 *Hae*II A fragment (29) isolated from either pDMS1020-6 or pDMS1043-6 with a colE1 *Hae*II E fragment (29) isolated from pDMS6642 (see Fig. 5).

Media. LS broth and LS agar (15) was used as a standard nutrient growth medium. M9 minimal medium (9) was supplemented with 5 μg of thymine per ml, appropriate amino acid supplements (40 μg/ml), or 0.4% Casamino Acids (Difco Laboratories). It was used for radioactive labeling of plasmid DNA. The concentrations of antibiotics were 100 μg of ampicillin and 5 μg of tetracycline per ml.

Isolation and purification of plasmid DNA. Plasmid DNA was released from cells by the cleared lysis procedure in which Triton X-100 was used exactly as previously described (9, 14). The covalently closed circular plasmid DNA was banded in CsCl-ethidium bromide density gradients and isolated exactly as previously described (9). The plasmid DNA was dialyzed against an appropriate buffer, phenol extracted, alcohol precipitated, and suspended in an appropriate buffer. The plasmid DNA was subsequently examined by agarose gel electrophoresis (9).

Hydroxylamine mutagenesis and selection of mutants affecting ColE1-mediated replication of pHSG124 DNA. Purified pHSG124 plasmid DNA, which confers ampicillin and tetracycline resistance and colicin E1 immunity on its host, was treated with hydroxylamine exactly as previously described (11), and the mutagenized DNA was used to transform P678-54. The transformation procedure is the slightly modified calcium-shock procedure of Mandel and Higa (15, 26). Selection of transformants containing a plasmid exhibiting a temperature-sensitive instability was achieved by plating the transformed cells after 2 h of incubation at 30°C in LS broth on LS agar plates containing 100 μg of ampicillin and 5 μg of tetracycline per ml. The plates were incubated at 30°C for 6 to 8 h followed by a shift to 42°C for an additional 15 h. Small colonies or microcolonies were picked at this point or the plates were reincubated for an additional 8 h at 30°C, and small colonies or microcolonies that appeared were then picked. Those colonies are restreaked on LS agar containing ampicillin and tetracycline. Single colonies were retested for the loss of the plasmid by demonstrating the simultaneous loss of both ampicillin and tetracycline resistance after cell growth at 42°C on LS agar.

Enzymatic digestion of DNA. The endonucleases used for DNA digestions were *Eco*RI and *Hae*II, which were used as previously described (10), and *Pst*I (New England Biolabs), which was used in accordance with the instructions accompanying the enzyme.

Agarose gel electrophoresis of DNA and elution of DNA from gels. Plasmid DNA digested with appropriate endonucleases was electrophoresed through agarose gels containing ethidium bromide as previously described (10). Agarose containing a particular DNA fragment was crushed in a polyalomer tube with an equal volume of elution buffer (0.5 M NH_4 acetate, 10 mM Mg acetate, 0.1 mM EDTA, and 0.1% sodium dodecyl sulfate), and the suspension was incubated overnight at 37°C. The agar suspension was centrifuged in an SW50.1 rotor for 1 h at 175,000 × *g*. The supernatant was phenol extracted, filtered through a Millipore HA membrane filter, alcohol precipitated, and suspended in an appropriate buffer for further study.

Ligation of endonuclease-cleaved fragments. Ligations were performed with T4 DNA ligase obtained from New England Biolabs, exactly as previously described (9, 16).

Transformation of bacteria with DNA. The method used for transformation is exactly as previously described (15, 26).

Determination of plasmid copy number. Cells were labeled for about two generations (2 h) with [^{3}H]thymidine, and cellular DNA was analyzed to determine the percentage of total cellular DNA existing as covalently closed circular molecules in CsCl-propidium diiodide density gradients exactly as previously described (9, 10). Cells were lysed by using Sarkosyl NL30. DNA-DNA membrane filter hybridization was also used to measure the percent of total cellular DNA that hybridizes to membrane-bound plasmid DNA. The method used was exactly as described previously (10) except that heat-denatured pDMS630 DNA was fixed to the membrane filters.

Measurement of conjugational transfer of ColE1 type plasmids. The Hfr H5 Sm^s donor strains carrying the plasmids to be tested and P678-54 Sm^r/E1 recipients were grown separately in LS broth at 30°C until they reached 2 × 10^8 cells per ml. Donor and recipient cells were mixed in equal amounts and incubated for 3 h at 30°C. The mating mixtures were diluted, conjugating pairs were disrupted, and the cells were plated on LS agar with appropriate antibiotics (19). Controls measuring transfer of pDMS630 or pHSG124 plasmid were done whenever transfer of presumptive mutants was measured.

Measurement of plasmid stability. A plasmid-containing colony was picked from an appropriate selective agar and suspended in LS broth, and the cultures were incubated at either 30 or 42°C. These cultures were periodically diluted and grown for about 60 generations. The bacterial viable counts after each dilution were determined on both LS agar and LS agar containing ampicillin or tetracycline or both that was

incubated at 30°C. Colonies growing on LS agar were also individually tested for drug resistance and colicin E1 immunity by picking cells from colonies with a toothpick and streaking them from the periphery of an LS agar plate into a central zone about 2.5 cm in diameter containing either antibiotic or colicin. Failure of cells to grow in that zone was interpreted as drug or colicin sensitivity. Appropriate controls were always performed. A colicin E1-immune cell was defined as one that is insensitive to the action of colicin E1 and sensitive to the action of colicin E2.

Electron microscopic analysis of DNA. The formamide procedure of Davis et al. (6), as previously described (13), was used.

RESULTS

Mutations of the pDMS630 component of pHSG124 that block pHSG124 replication were sought by mutagenizing pHSG124 DNA in vitro with hydroxylamine (11). The mutagenized DNA was used to transform *E. coli*, and transformants were selected that exhibited a temperature-sensitive loss of both ampicillin and tetracycline drug resistance as described above. Plasmids isolated from 4 out of 12 transformants that exhibited an unstable drug resistance phenotype were further examined. Those plasmids were called pDMS1020, pDMS1037, pDMS1043, and pDMS1050.

Plasmid stability. The kinetics of the loss of the mutant plasmids from P678-54 cells grown in LS broth at 42°C is shown in Fig. 1. The results were identical when the plasmids were transferred by transformation to other P678-54 cells or to Om84 cells and tested in the same way (data not shown). The results suggest that the mutations affect the pDMS630-mediated replication or segregation process in the chimeras and are not host specific.

The pDMS630 components of each of these mutant chimeras were reisolated to determine whether their replication and maintenance was affected. DNA of each chimeric mutant was digested with *Eco*RI endonuclease, and the digests were then religated, thus reforming both the pDMS630 and the pHSG1 plasmid components (9). The religated DNA was used to transform P678-54. Ampicillin-resistant, tetracycline-sensitive transformants containing plasmid DNA that is identical in size to pDMS630 and exhibiting the pDMS630 *Hae*II endonuclease fragment pattern were obtained (data not shown). The mutations, therefore, clearly do not cause absolute replication defects in the pDMS630 components of the chimeric mutants and do not exhibit any large deletions. The pDMS630 components of pDMS1020, pDMS1037, pDMS1043, and pDMS1050 isolated in this way were named, respectively, pDMS1020-6, pDMS1037-6, pDMS1043-6, and pDMS1050-6. The mutants were all found to be identical in size to pDMS630 and to exhibit identical *Hae*II endonuclease fragment patterns, which suggests they do not contain large deletions.

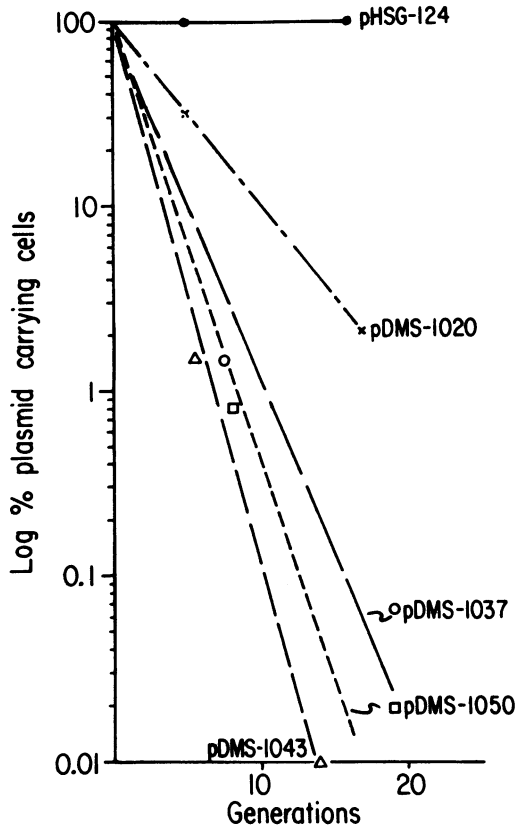

FIG. 1. *Stability of the plasmid-associated antibiotic resistance phenotype in P678-54 during growth at 42°C. Single colonies containing the plasmids pDMS1020, pDMS1037, pDMS1043, pDMS1050, or the parental control, plasmid pHSG124, were picked from LS agar containing ampicillin and tetracycline, suspended in LS broth, and incubated at 42°C. Cells were diluted at intervals and plated on either LS or LS-ampicillin-tetracycline agar, which was then incubated at 30°C.*

Whereas the chimeric plasmid mutants could be stably maintained at 30°C (data not shown), the pDMS630 components could not. The plasmid instability at 30°C was examined by picking a single plasmid-carrying colony from ampicillin agar and growing the cells in LS broth. The viable counts were periodically determined on LS and LS plus ampicillin agar (Fig. 2). pDMS1020-6 was the most stable mutant but was lost over longer periods of growth in the absence of ampicillin. Attempts to study the kinetics of segregation of pDMS1020-6,

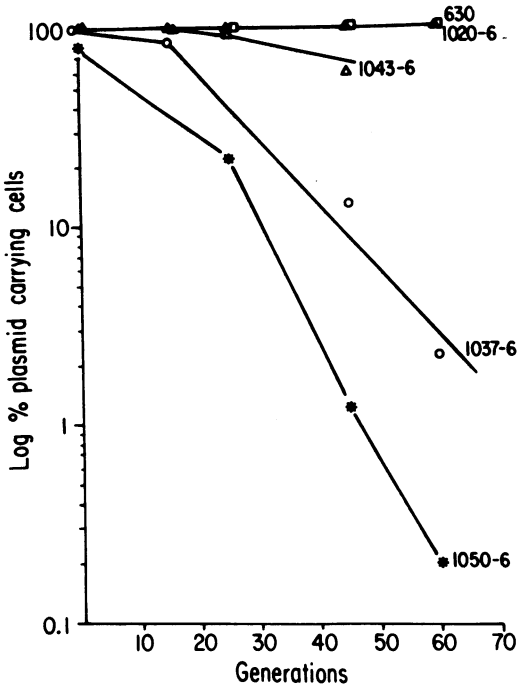

FIG. 2. *Stability of the pDMS630 components isolated from chimeric plasmid mutants. The percentage of plasmid-carrying, ampicillin-resistant cells in a culture grown in LS broth at 30°C for about 60 generations is shown.*

pDMS1037-6, pDMS1043-6, and pDMS1050-6 in cells grown at 42°C in LS broth were unsuccessful, since all plasmid-carrying cells exhibited an abrupt but variable loss of viability after the temperature shift. Survival of from 10 to 0.1% of the cells was observed with different mutants. It was clearly shown, however, that many of the cells that survived and grew at 42°C had lost the plasmid. It is known that the induction of colicin E1 synthesis and cell death is related to shifts of cells to high temperatures (22).

Plasmid copy number. The copy number of pHSG124 and similar plasmids (2) more closely resembles that of pDMS630 (about 15 copies per chromosome) than that of pHSG1 (about 4 copies per chromosome) (9). A mutation that interferes with pDMS630 replication in the chimera should lead to a reduced chimera copy number. The plasmid copy numbers were determined by growing P678-54 *thy* cells containing the respective chimeras overnight in the presence of antibiotic, diluting them 1/20 in M9 medium and growing them for 2 h before labeling the DNA for about two generations in the presence of [^{3}H]thymidine at 30 or 42°C and measuring the percentage of the total radioactivity in the cellular DNA that banded in a CsCl-propidium diiodide density gradient (9) as covalently closed circular, replicative-intermediate, or catenated plasmid DNA (17). The percentage of labeled cells that carried plasmid was determined by plating cells on LS agar with or without antibiotic. Of cells grown in antibiotic-containing medium before labeling, 100% contained plasmid at the time of labeling. Compared with pHSG124, pDMS1020 had about 0.5, and pDMS1037, pDMS1043, and pDMS1050 had about 0.3, times the number of copies per chromosome in cells labeled at both 30 (Fig. 3) and 42°C (gradients not shown). Since the chimeras were stably maintained at 30°C, the reduced plasmid copy number obtained upon labeling at 30°C is suggestive of a compromised plasmid replication capability. The result also indicates that the pDMS630 component of the plasmid is not totally blocked in its replication in the chimera at 42°C during the labeling period.

The copy number of pDMS1020-6, pDMS1037-6, pDMS1043-6, and pDMS1050-6 was also determined by both membrane filter DNA-DNA hybridization and CsCl-propidium

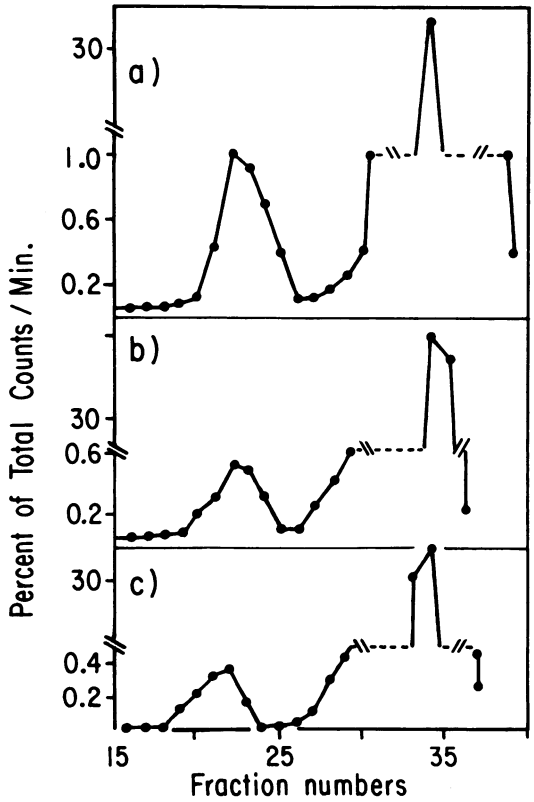

FIG. 3. *Cesium chloride-propidium diiodide gradients containing total cellular DNA that was isolated from plasmid-carrying P678-54 cells. (a) pHSG124; (b) pDMS1020; (c) pDMS1050. Each sample contained about 100,000 cpm.*

diiodide centrifugation analysis. The results of the membrane filter hybridization experiments with DNA labeled at 30 and 42°C (Table 2) show that there were about 14 copies of pDMS630 DNA per host chromosome at both temperatures. The mutants pDMS1037-6, pDMS1043-6, and pDMS1050-6 had about half the wild-type copy number of 30°C and between one-quarter and one-half the wild-type copy number at 42°C. It is clear that there was not a rapid or preferential shutoff of plasmid DNA replication during [^{3}H]thymidine labeling at 42°C since that would lead to a much reduced apparent copy number compared with the results obtained by labeling at 30°C. There may, however, be a reduction of some mutant plasmid replication at 42°C. The surprising result was the high copy number exhibited by pDMS1020-6 at both 30 and 42°C. CsCl-propidium diiodide centrifugation analysis of the total cellular DNA labeled at 30°C confirmed the hybridization results. pDMS1020-6 had a copy number about four times greater than that of pDMS630, whereas pDMS1037-6, pDMS1043-6, and pDMS1050-6 had, respectively, 0.5, 0.5, and 0.3 times the copy number of pDMS630.

TABLE 2. *Membrane filter DNA-DNA hybridization determination of plasmid content of cells at 30 and 42°C*[a]

Plasmid	Temp (°C)	Total cpm added	% of total cpm bound	Copy no. as fraction of pDMS630
pDMS1020-6	30	10,130	10.5	3.2
	42	1,340	16.7	5.6
pDMS1037-6	30	35,188	1.65	0.50
	42	59,148	1.29	0.43
pDMS1043-6	30	65,720	1.58	0.48
	42	50,922	0.72	0.24
pDMS1050-6	30	157,374	1.35	0.45
	42	135,102	1.05	0.30
pDMS630	30	76,140	3.3	1.0
	42	39,240	3.0	1.0

[a] Sonicated, heat-denatured, [^{3}H]thymidine-labeled cellular DNA was incubated with membrane filters containing 5 μg of pDMS630 DNA. The filters were subsequently washed and incubated, and the bound labeled DNA was measured (10). Bacterial DNA that nonspecifically bound to filters (0.2%) was substracted from all samples. Of the radioactively labeled pDMS630 DNA, 32% bound to filters containing pDMS630 DNA. A correction to 100% binding of added pDMS630 DNA was calculated to give the percentage of total counts per minute bound that is pDMS630 or pDMS630 mutant DNA. Assuming a bacterial chromosome size of 3.0×10^9 daltons, the copy number of pDMS630, which is 7.0×10^6 daltons in size, is 14 at 30°C in this experiment.

Replication in the presence of chloramphenicol. The replication process used to replicate ColE1 DNA may not be identical in the presence (3) and absence of chloramphenicol. The ability of the mutant chimeras and the pDMS630 mutants isolated from them to replicate in the presence of the drug at 30 and 42°C was examined. The accumulation of large amounts of covalently closed circular plasmid DNA in cells incubated in the presence of chloramphenicol was determined by directly examining CsCl-ethidium bromide density gradients containing either the Triton X-100 cleared lysates (3) or total Sarkosyl lysates (9) of cells that had been incubated for 15 h in the presence of chloramphenicol. Both lysis procedures were used since failure to observe an accumulation of covalently closed circular DNA in cleared lysates could be caused by the trapping of an unusual replicative intermediate in the cell or chromosome component of the cleared lysate. Samples (10 ml each) of cultures containing about 3×10^8 plasmid-carrying cells per ml were incubated at 30°C in the presence of 180 μg of chloramphenicol per ml for 15 h and lysed. The results of Sarkosyl lysis of those cultures (Fig. 4) indicate that the copy number of pHSG124 and pDMS1043 was greatly increased, whereas a much smaller increase was seen in the accumulation of pDMS1037 and pDMS1050. The copy number of pDMS630 and pDMS1043-6 was also greatly increased under similar conditions, whereas pDMS1037-6 and pDMS1050-6 showed little increase in copy number (data not shown). The preparations of cleared lysates produced similar results for these plasmids. The results of examining the replication of the plasmids in the presence and absence of chloramphenicol suggest that the replication capability of pDMS1037, pDMS1037-6, pDMS1050, and pDMS1050-6 was greatly reduced compared with that of pHSG124 or pDMS630. P678-54 and Om84 host cells were used to insure that alterations in plasmid replication was not host-cell specific. In all experiments, essentially 100% of the cells were ampicillin resistant at the time of addition of chloramphenicol and therefore presumably carried the plasmid. The DNA in the upper band of each gradient (Fig. 4), which could possibly contain significant amounts of nicked circular DNA as well as linear bacterial DNA, was isolated and examined on agarose gels after *Eco*RI endonuclease digestion to distinguish plasmid from DNA. Significant amounts of open circular DNA would yield a unique band

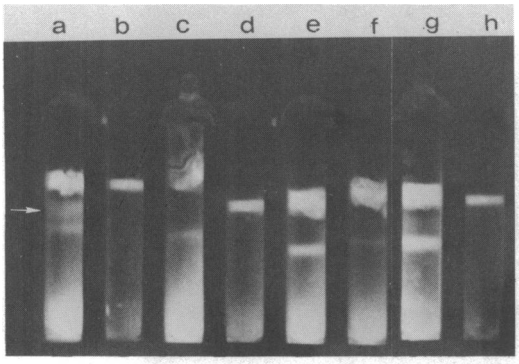

FIG. 4. *Cesium chloride-ethidium bromide density gradients containing plasmid DNA released from cells that have been incubated in the presence or absence of chloramphenicol. The host bacteria was P678-54 in all cases shown. Cells were lysed with Sarkosyl. Cells were incubated for 15 h with chloramphenicol (+). Cells were lysed at the time of addition of chloramphenicol (−). (a) pDMS1020 (+); (b) pDMS1020 (−); (c) pDMS1037 (+); (d) pDMS1037 (−); (e) pDMS1043 (+); (f) pDMS1050 (+); (g) pHSG124 (+); (h) pHSG124 (−). The bacterial viable counts and numbers of plasmid-carrying cells in all cultures were essentially identical when chloramphenicol was added. Arrow indicates intermediate density fraction. The lowest band is covalently closed circular molecules.*

on the gels rather than a simple smear pattern if only bacterial DNA was present. In no instance were significant amounts of plasmid DNA recovered from those bands.

pDMS1020 (Fig. 4) and pDMS1020-6 (data not shown) exhibited an accumulation of plasmid DNA at 30°C in the lower band that corresponded to covalently closed circular DNA but also exhibited considerable accumulation of DNA at a postition between covalently closed circular and linear or open circular DNA. Those reisolated and repurified molecules obtained from the intermediate density position were examined by electron microscopy and found to contain obviously catenated structures which are not frequently generated by ColE1 (17) as well as clusters of plasmid molecules (Fig. 5). Cairn's-type replicative intermediates were not seen (17). The presence of the clusters of molecules were unaffected by pronase or phenol treatment or by a reduction in the DNA concentration in preparations used for electron microscopy. Clusters are presently thought to represent multimeric catenated structures. The catenated molecular forms of pDMS1020 or pDMS1020-6 were not usually observed in cleared lysates (data not shown). Occasionally, when some were seen, they were present at levels disproportionately lower than what was observed in Sarkosyl lysates of the same culture.

It was found that neither ColE1- nor ColE1-derived plasmids accumulated in cells incubated in the presence of chloramphenicol at 42°C (data not shown). ColE1 exhibited normal amplification of copy number at 30 and 37°C. A test of the reversibility of the temperature-sensitive block of ColE1 replication in the presence of chloramphenicol was performed by incubating the cells for varying periods at 42°C in the presence of chloramphenicol and then shifting them to 30°C for 15 h before lysing them. The results showed that the accumulation of plasmid in cells was markedly reduced after incubation at 42°C for between 3 and 6 h before cells were returned to 30°C (data not shown). The replication inhibition at 42°C was clearly reversible over shorter periods.

Localization of mutations in the *Hae*II A or E fragments of ColE1. Attempts to localize the various mutations to that part of the pDMS630 plasmid containing the ColE1 *rep* region (27, 30) have been only partially successful. The plasmid *rep* region (27) (Fig. 6) is in the *Hae*II A and E fragments of ColE1. The *Hae*II A fragment also carries the colicin immunity gene. The *Hae*II A fragments of pDMS630, pDMS1020-6, pDMS1037-6, pDMS1043-6, and pDMS1050-6 have been isolated from the respective mutants and ligated to the *Hae*II E fragment of the ColE1 plasmid derivative pDMS6642 to form new small plasmids. Since pDMS6642 does not have the colicin E1 immunity gene, newly formed plasmids conferring colicin immunity must have acquired the *Hae*II A fragment from the mutant plasmid. Such plasmids will not contain the A region of pDMS6642 if they fail to confer ampicillin resistance on their host. The combination of a *Hae*II A and E fragment produces a stable plasmid unless a genetic defect is contained in the information carried by one fragment. P678-54 was transformed with a ligation mixture, and colicin-immune transformants were selected. Those that were ampicillin sensitive were examined further. (No transformants were obtained when the separate DNAs were ligated and used in the transformation). The DNA from those cells was isolated and analyzed on agarose gels. Four of four transformants tested contained an *Hae*II A fragment of pDMS1020-6 ligated to the *Hae*II E fragment of pDMS6642, and three of four transformants contained a *Hae*II A fragment of pDMS1043-6 ligated to the *Hae*II E fragment of pDMS6642. Only one orientation of the *Hae*II A and E fragments in ColE1 plasmids is possible if those fragments are the only source of the

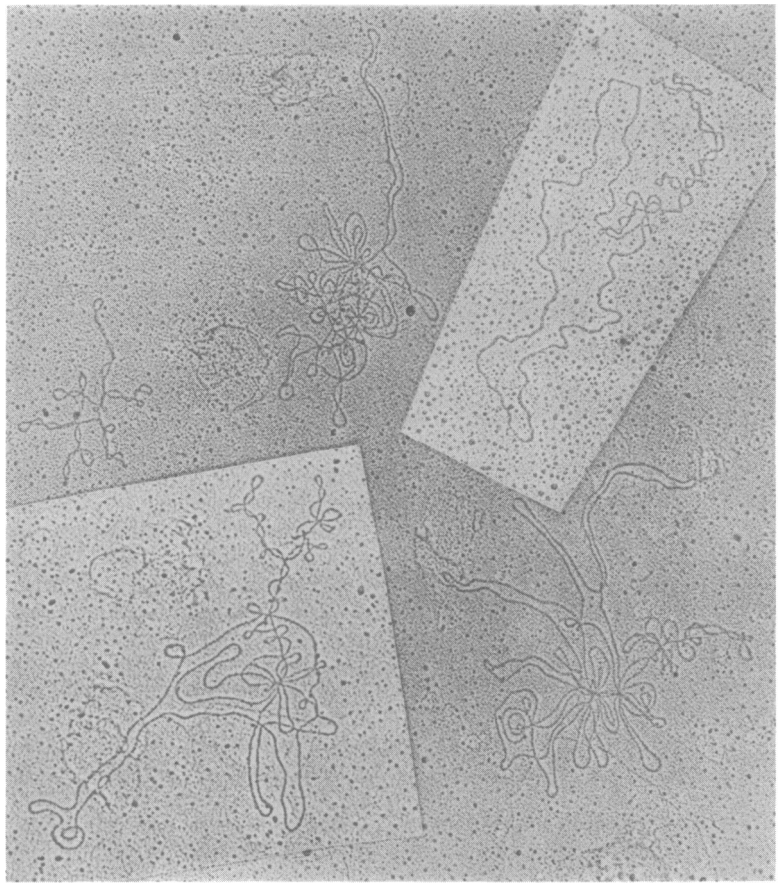

FIG. 5. *Electron micrographs of catenated and clustered DNA molecules isolated from pDMS1020 (Fig. 3b).*

plasmid *rep* region (16). These plasmids were referred to as pDMS1020-7 and pDMS1043-7, respectively. *Hae*II digests of pDMS1020-7 and 1043-7 produced the expected *Hae*II A and E fragments.

The stability of pDMS1020-7 or pDMS1043-7 was tested in cells grown in LS broth at 30 and 42°C for about 40 generations. The plasmid pDMS1020-7 was slightly unstable at 30°C (between 20 and 30% of the cells lost the plasmid), whereas at 42°C, more than 95% of the cells tested lost the plasmid. That result suggests that the presence of a *Hae*II A fragment from pDMS1020-6 confers an instability on the newly formed plasmid. The plasmid pDMS1043-7 was completely stable at both temperatures. The results suggest that the mutation in pDMS1043-6 that leads to plasmid instability at 42°C (Fig. 1) is probably not in the *Hae*II A fragment but possibly in the *Hae*II E fragment. Three attempts to isolate colicin-immune plasmids by ligating the *Hae*II A fragment of either pDMS1037-6 or pDMS1050-6 to a *Hae*II E fragment of pDMS6642 have been unsuccessful. Several colicin E1-immune, ampicillin-sensitive colonies were obtained. However, in only one instance was a small unstable plasmid corresponding in size to plasmids containing only a *Hae*II A and E fragment isolated from transformants. The ligation reactions with *Hae*II A fragments from either pDMS1037-6 or pDMS1050-6 were essentially identical to reactions with the *Hae*II A fragments from the other mutants in terms of the amounts of DNA used. The efficiency of the ligation reactions and recipient cells used for transformation were also the same. These negative results were therefore not considered trivial but were taken to suggest that the mutations affecting plasmid stability in pDMS1037-6 and pDMS1050-6 were in the *Hae*II A fragment of those plasmids (see below).

Conjugational transfer of plasmid mutants. It was reported that a temperature-sensitive replication-defective mutant of a ColE1

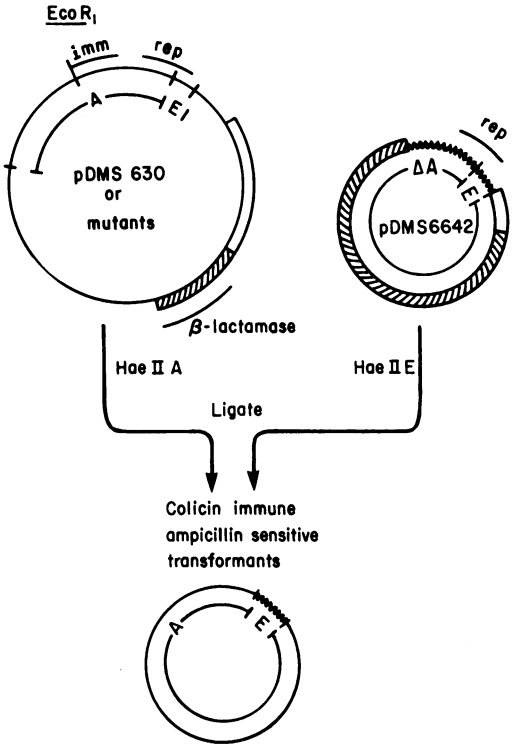

FIG. 6. *Diagramatic representation of the test of the location of mutations in the HaeII A or E fragments of ColE1. The elements shown on the pDMS630 plasmid or the mutants 1020-6, 1037-6, 1043-6, and 1050-6 are the immunity gene (imm), the replication region (rep), and the β-lactamase gene (▨) contained in the Tn3 transposon (□). HaeII endonuclease-sensitive sites marking the ends of the HaeII A and E fragments (1) of ColE1 DNA are shown. The EcoRI-sensitive site is shown. The pDMS6642 plasmid contains the HaeII E fragment and part of the HaeII A fragment (ΔA) of ColE1 and the β-lactamase gene and part of the Tn3 transposon fused to the ΔA fragment. There are only two HaeII endonuclease-sensitive sites in pDMS6642. The HaeII A fragment of pDMS630 or its mutants and the HaeII E fragment derived from pDMS6642 were ligated, and the stability of the resulting plasmids was examined.*

derivative also exhibited a temperature-sensitive relaxation effect (4). The possibility that the mutations occurring in these plasmids affect plasmid relaxation was indirectly tested by examining the frequency of conjugational transfer of pDMS1020-6, pDMS1037-6, pDMS1043-6, or pDMS1050-6 to determine whether transfer was significantly reduced. The approach was used since all mutations so far studied in this laboratory that fail to exhibit relaxation also exhibit at least a 100-fold-reduced conjugational transfer (14, 15, 19). If the efficiency of transfer of pDMS630 is taken as 1 (19 and above), then pDMS1020-6, pDMS1020-6, pDMS1037-6, pDMS1043-6, and pDMS1050-6 are transferred with an efficiency of 2.0, 0.60, 0.45, and 0.25 times, respectively, the efficiency of pDMS630. The results, therefore, suggest that the mutations do not affect the plasmid relaxation process.

DISCUSSION

Hydroxylamine-mutagenized chimeric pHSG124 DNA was used to transform *E. coli*. A procedure was devised to identify transformants carrying plasmids that exhibit instability when the replication of the chimeric plasmids was solely dependent on the ColE1 component. The chimeric plasmids isolated from those transformants showed alterations in their stability, copy number, capacity to replicate, or structure after replicating in the presence of chloramphenicol. The chimeras' mutant properties were found to be exhibited by the ColE1 component (pDMS630) of the chimeras. Since the induced mutations are probably transition mutations (7) the further analysis of these mutants should provide information not obtainable from studies of insertion and deletion mutations about the organization of ColE1 genetic information that affects plasmid stability and replication.

Evidence was presented that demonstrated that the mutation in pDMS1020-6 and probably in pDMS1037-6 and pDMS1050-6 resides in the *Hae*II A fragment of the plasmid. This is not unexpected since the replication region of ColE1 (27) and information affecting replication, copy number, and incompatibility (1, 5, 10, 12, 16, 20, 23, 27) has been found to reside in part of that fragment. Evidence indicated that the mutation affecting pDMS1043-6 is not located in the *Hae*II A fragment of ColE1 DNA. Since the remainder of the ColE1 replication region resides in the *Hae*II E fragment of the plasmid, it seems likely that the pDMS1043-6 plasmid mutation will be identified there, although mutations affecting the *Hae*II C fragment of ColE1 have been linked to alterations in plasmid copy number (31).

The failure to obtain colicin E1-immune transformants from mixtures of *Hae*II A fragments derived from pDMS1037-6 and *Hae*II E fragments derived from pDMS6642 has been interpreted to mean that a mutation in pDMS1037-6 resides in that plasmid *Hae*II A fragment. Bacterial colonies containing pDMS1037-6 generally exhibit a lower copy number than pDMS630 and a correspondingly greater sensitivity to ampicillin. The level of ampicillin to which a cell carrying the ColE1 plasmid-coded Tn*3* β-lactamase gene is resistant correlates with the plasmid

copy number (9, 10). pDMS1037-6 containing cells were also found to exhibit an increased sensitivity to colicin. In a population of ampicillin-resistant colonies carrying pDMS1037-6, 60% of the colonies tested showed little or no colicin immunity. This presence of ampicillin resistance and increased sensitivity to colicin is explainable if the reduced copy number of pDMS1037-6 in those cells causes a reduction in the amount of colicin immunity substance produced per cell and if the level of colicin E1 used to select the transformants on agar overwhelms this immunity. Several previously reported observations indirectly support this interpretation. It is known that colicin immunity can, in some cases, be overwhelmed by the addition of excess colicin in the medium (25). In the colicin E3 system, the colicin molecule and immunity substance copurify, suggesting a direct interaction. It has also been shown that equimolar amounts of colicin E3 immunity substance added to colicin E3 blocks the colicin action in vitro (21).

pDMS1020 and pDMS1020-6 produced significant levels of catenated DNA when cells were incubated in the presence of chloramphenicol. Catenated ColE1 DNA is only found in low levels in pDMS630 or other ColE1 plasmid-containing *E. coli* cells (17). Sakakibara et al. (28) found that ColE1 catenate formation in vitro is a consequence of events that occur during the terminal stage of replication. It is therefore possible that the mutation carried by pDMS1020-6 affects a step at the termination of replication. The finding that the catenated structures produced by pDMS1020-6 are only found in significant numbers when cells are lysed with Sarkosyl rather than by the cleared lysis procedure suggests that the association of the catenated plasmids with cell structures is different from that of noncatenated molecules. Since the mutation carried by pDMS1020-6 causes the plasmid to be more unstable than pDMS630, there may be a correlation between the mutants' instability and the formation or localization or both of catenated structures. The observation that pDMS1020-7 is also unstable suggests that the mutation affecting the plasmid is in the *Hae*II A fragment of the plasmid. Since replication termination of ColE1 occurs adjacent to the replication origin, in the *Hae*II E fragment (28), the mutation is probably not precisely at the replication termination site but before it.

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LITERATURE CITED

1. **Backman, K., M. Betlach, H. W. Boyer, and S. Yanofsky.** 1978. Genetic and physical studies on the replication of ColE1-type plasmids. Cold Spring Harbor Symp. Quant. Biol. **43**:69–70.
2. **Cabello, F., K. Timmis, and S. N. Cohen.** 1976. Replication control in a composite plasmid constructed by *in vitro* linkage of two distinct replicons. Nature (London) **259**:285–290.
3. **Clewell, D. B.** 1972. Nature of ColE1 plasmid replication in *Escherichia coli* in the presence of chloramphenicol. J. Bacteriol. **110**:667–676.
4. **Collins, J., S. Yanofsky, and D. R. Helinski.** 1978. Altered DNA-protein relaxation complex in a replication mutant of plasmid ColE1. Mol. Gen. Genet. **167**: 21–28.
5. **Conrad, S. E., and J. L. Campbell.** 1979. Role of plasmid-coded RNA and ribonuclease III in plasmid DNA replication. Cell **18**:61–71.
6. **Davis, R. W., M. Simon, and N. Davidson.** 1971. Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. Methods Enzymol. **21**:413–428.
7. **Freese, E., E. Bautz-Freese, and E. Bautz.** 1961. The chemical and mutagenic specificity of hydroxylamine. Proc. Natl. Acad. Sci. U.S.A. **47**:845–849.
8. **Gelfand, D. H., H. M. Shepard, P. H. O'Farrell, and B. Polisky.** 1978. Isolation and characterization of ColE1-derived plasmid copy-number mutant. Proc. Natl. Acad. Sci. U.S.A. **75**:5869–5873.
9. **Hashimoto-Gotoh, T., and J. Inselburg.** 1979. Isolation and characterization of replication-deficient mutants of ColE1 plasmids. J. Bacteriol. **139**:597–607.
10. **Hashimoto-Gotoh, T., and J. Inselburg.** 1979. ColE1 plasmid incompatibility: localization and analysis of mutations affecting imcompatibility. J. Bacteriol. **139**: 608–619.
11. **Hashimoto-Gotoh, T., and M. Sekiguchi.** 1977. Mutations to temperature sensitivity in R plasmid pSC101. J. Bacteriol. **131**:405–412.
12. **Hefron, F., M. So., and B. J. McCarthy.** 1978. *In vitro* mutagenesis of a circular DNA molecule by using synthetic restriction sites. Proc. Natl. Acad. Sci. U.S.A. **74**: 1865–1869.
13. **Inselburg, J.** 1973. Colicin factor DNA: a single nonhomologous region in ColE2-E3 heteroduplex molecules. Nature (London) New Biol. **241**:234–237.
14. **Inselburg, J.** 1977. Isolation, mapping, and examination of effects of TnA insertions in ColE1 plasmids. J. Bacteriol. **129**:482–491.
15. **Inselburg, J.** 1977. Studies of colicin E1 plasmid functions by analysis of deletions and TnA insertions of the plasmid. J. Bacteriol. **132**:332–340.
16. **Inselburg, J.** 1980. Functional relationship between parts of the replication region of plasmid ColE1. J. Bacteriol. **143**:1073–1076.
17. **Inselburg, J., and M. Fuke.** 1971. Isolation of catenated and replicating DNA molecules of colicin factor E1 from minicells. Proc. Natl. Acad. Sci. U.S.A. **68**:2839–2842.
18. **Inselburg, J., and P. Ware.** 1977. Isolation and genetic analysis of deletion mutants of colicin E1 plasmids carrying a TnA insertion. J. Bacteriol. **132**:321–331.
19. **Inselburg, J., and P. Ware.** 1979. A complementation analysis of mobilization deficient mutants of the plasmid ColE1. Mol. Gel. Genet. **172**:211–219.
20. **Itoh, T., and J. Tomizawa.** 1980. Formation of an RNA primer for initiation of replication of ColE1 DNA by ribonclease H. Proc. Natl. Acad. Sci. U.S.A. **77**:2450–2454.
21. **Jakes, K., N. D. Zinder, and T. Boon.** 1974. Purification and properties of colicin E3 immunity protein. J. Biol. Chem. **249**:438–444.
22. **Kennedy, C. K.** 1977. Induction of colicin production by high temperatures or inhibition of protein synthesis. J. Bacteriol. **108**:10–19.
23. **Kingsbury, D. T., and D. R. Helinsky.** 1973. Temper-

ature-sensitive mutants for the replication of plasmids in *Escherichia coli*. Isolation and specificity of host and plasmid mutants. Genetics **74**:17–31.

24. **Kingsbury, D. T., D. G. Sieckmann, and D. R. Helinski.** 1973. Temperature-sensitive mutants for the replication of plasmids in *Escherichia coli*. II. Properties of host and plasmid mutations. Genetics **74**:1–16.
25. **Levisohn, R., J. Konisky, and M. Nomura.** 1968. Interaction of colicins with bacterial cells. IV. Immunity breakdown studied with colicins Ia and Ib. J. Bacteriol. **96**:811–821.
26. **Mandel, M., and A. Higa.** 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53**:159–162.
27. **Oka, A., N. Nomura, M. Morita, H. Sugisaki, K. Sugimoto, and M. Takanami.** 1979. Nucleotide sequences of small ColE1 derivatives: structure of the regions essential for autonomous replication and colicin E1 immunity. Mol. Gen. Genet. **172**:151–159.
28. **Sakakibara, Y., K. Suzuki, and J. Tomizawa.** 1976. Formation of catenated molecules by replication of colicin E1 plasmid DNA in cells extracts. J. Mol. Biol. **108**:569–582.
29. **Shepard, H. M., D. H. Gelfand, and B. Polisky.** 1979. Analysis of a recessive plasmid copy number mutant: evidence for negative control of ColE1 replication. Cell **18**:267–275.
30. **Tomizawa, J., H. Ohmori, and R. E. Bird.** 1977. Origin of replication of colicin E1 plasmid DNA. Proc. Natl. Acad. Sci. U.S.A. **72**:1865–1869.
31. **Twigg, A. J., and D. Sherratt.** 1980. Trans-complementable copy-number mutants of plasmid ColE1. Nature (London) **283**:216–218.